Elevated Oral and Systemic Levels of Soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1) in Periodontitis

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Abstract: The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is a cell-surface receptor of the immunoglobulin superfamily, involved in the propagation of the inflammatory response to bacterial challenge. Soluble (s)TREM-1 is released from the cell surface during the course of infection and is a useful inflammatory biomarker in the early diagnosis of systemic sepsis. The hypothesis of this study was that oral and systemic levels of sTREM-1 are elevated in periodontitis. Therefore, the aim was to investigate, by ELISA, the sTREM-1 concentrations in saliva and serum of individuals without periodontitis (control) and persons with chronic or generalized aggressive periodontitis. In saliva, sTREM-1 concentrations were higher in chronic and aggressive periodontitis than in the control group, by 3.3-fold and 5.6-fold, respectively. In serum, these differences were 1.7-fold and 2-fold, respectively. However, there were no significant differences between the two forms of periodontitis, neither in saliva nor in serum. Salivary and serum sTREM-1 levels positively correlated with full-mouth clinical periodontal parameters. In conclusion, the increased oral and systemic levels of sTREM-1 in periodontitis denote a value for this molecule as a biomarker for the disease and may also have implications in the association between periodontal infections and systemic inflammatory response.

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ABSTRACT

The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily, involved in the propagation of the inflammatory response to bacterial challenge. Soluble (s)TREM-1 is released from the cell surface during the course of infection, and is a useful inflammatory biomarker in the early diagnosis of systemic sepsis. The hypothesis of this study is that oral and systemic levels of sTREM-1 are elevated in periodontitis. Therefore, the aim was to investigate by ELISA the sTREM-1 concentrations in saliva and serum of non-periodontitis subjects (control) and patients with chronic or generalized aggressive periodontitis. In saliva, sTREM-1 concentrations were higher in chronic and aggressive periodontitis than the control group, by 3.3-fold and 5.6-fold, respectively. In serum, these differences were 1.7-fold and 2-fold, respectively. However, there were no significant differences between the two forms of periodontitis, neither in saliva nor in serum. Salivary and serum sTREM-1 levels positively correlated with full mouth clinical periodontal parameters. In conclusion, the increased oral and systemic levels of sTREM-1 in periodontitis denote a value for this molecule as a biomarker for the disease, and may also have implications in the association between periodontal infections and systemic inflammatory response.

INTRODUCTION

The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily, involved in the innate inflammatory response to bacterial and fungal infections (Bleharski et al., 2003). Early studies have demonstrated the role of TREM-1 the development of septic shock (Begum et al., 2004; Bouchon et al., 2001). It was further demonstrated that the systemic production of TREM-1 is enhanced in infections of the respiratory track, gut or the amniotic fluid (Begum et al., 2004; Buckland et al., 2011). TREM-1 is produced primarily by monocytes (Arts et al., 2011; Cavaillon, 2009) and regulates immune cell functions in a manner that leads to the enhancement of the inflammatory response (Colonna and Facchetti, 2003; Ford and McVicar, 2009). The engagement of TREM-1 in *in vivo* and *in vitro* models by agonist monoclonal antibodies further stimulates the production of pro-inflammatory cytokines (Bleharski et al., 2003; Bostanci et al., 2011; Bouchon et al., 2001; Radsak et al., 2004). A synergism exists between the activation of TREM-1 and toll-like receptors or Nod-like receptors, resulting in amplification of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and inhibition of anti-inflammatory cytokines such as IL-10 (Bleharski et al., 2003). Hence, the role of TREM-1 in innate immunity appears to be the regulation of the magnitude of the inflammatory response to bacterial challenge. Of relevance to periodontal disease is the finding that *Porphyromonas gingivalis* induces TREM-1 production in monocytes, characterised by a shift from the cell bound to its soluble form, along with the propagation of pro-inflammatory cytokine production (Bostanci et al., 2011; Liang et al., 2009). This effect can be diminished by the administration of doxycycline in the experimental system (Bostanci and Belibasakis, 2012).

As TREM-1 is released during the course of infection in the form of soluble (s)TREM-1, it can serve as a particularly useful marker of systemic inflammation. This has been well demonstrated in systemic sepsis (Gibot et al., 2004a; Gibot et al., 2004b; Su et al., 2012), arthritis (Collins et al., 2009; Murakami et al., 2007), pulmonary infections (Ruiz-Gonzalez et al., 2011), pancreatitis (Yasuda et al., 2008) and inflammatory bowel disease (Park et al., 2009). sTREM-1 is a biomarker that can easily be measured in biological fluids (Skogstrand et al., 2005). The presence of sTREM-1 in serum could be attributed to circulating leukocytes during the course of systemic infection, or could account for locally produced sTREM-1 in focal infections, which eventually enters the blood stream (Bleharski et al., 2003).

Even though the systemic involvement of sTREM-1 has been demonstrated in a number of infections, little is known about its association with periodontal disease. Thus the hypothesis of this study is that oral and systemic TREM-1 levels are elevated in periodontitis, and could constitute a systemic biomarker for the disease. Therefore, the aim of the study was to investigate the salivary and serum sTREM-1 levels, in subjects without periodontitis and patients with chronic or aggressive periodontitis.

MATERIALS AND METHODS

Study population and clinical examination

A total of 63 subjects were included in this study, recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir, Turkey, from November 2011 until March 2012. The use of human subjects satisfied the requirements of Ege University Institutional Review Board (ethics approval number: 11-12.1/11) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. It is confirmed that this cross-sectional study conforms to

STROBE guidelines for observational studies. Complete medical and dental histories were taken from all subjects. Systemic exclusion criteria were presence of cardiovascular and respiratory diseases, diabetes mellitus, HIV infection, systemic inflammatory conditions or non-plaque induced oral inflammatory conditions, immunosuppressive chemotherapy and current pregnancy or lactation. None of the patients had taken medication such as antibiotics or anti-inflammatory drugs that could affect their periodontal status for at least 6 months before the study. Patients eligible for the study returned to the clinic for clinical measurement screening, 1 week after pre-screening. Smoking status was also recorded, and participants smoking > 5 cigarettes per day were registered as smokers. Prior to enrolment in the study, written and informed consent was obtained from each subject. Patient selection was based on clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999). The clinical measures and patient demographics are described in the Appendix.

Collection and processing of saliva and serum

All clinical samples (saliva and serum) were collected in the morning between 8 a.m. and 11 a.m. Subjects were asked to avoid oral hygiene measures (i.e., flossing, brushing, and mouth rinses), eating, drinking for 2 h before collection. For saliva collection, the subjects asked to rinse their mouth with tap water, following which they expectorated whole saliva into sterile 50 ml tubes for 5 min. All saliva samples were placed on ice, supplemented with EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland) prior to centrifuging at 10000 x g for 15 min at 4°C (Navazesh, 1993). Finally, the resulting supernatants were then immediately aliquoted and frozen at - 80°C. For serum samples, 5 ml of venous blood were taken into

vacutainers (BD Diagnostics) by a standard venipuncture method. Once collected, the samples were left at room temperature to allow for blood clotting, and then centrifuged to remove the fibrin clot and cellular elements for 15 min at 1500 x g at 4°C. All serum samples were placed on ice, supplemented with EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland). Finally, they were immediately aliquoted and frozen at – 80 °C. Further analysis of the saliva and serum samples by ELISA is described in the Appendix.

Statistical analysis

Statistical analysis was performed using non-parametric methods. Comparisons between all groups were performed using the Kruskal–Wallis test. When significant differences were observed ($p<0.008$), then two-group comparisons were assessed with Mann–Whitney *U*-tests with Bonferroni correction, and $p<0.05$ was considered to be statistically significant. Correlations between sTREM-1 levels and clinical parameters were analyzed by Spearman's rank test and $p < 0.01$ values were considered as significant. Statistical analyses were conducted using the statistical software SPSS v. 19.0 (IBM, Somers, NY, USA).

RESULTS

Clinical findings

The full mouth clinical findings are provided in Table 1. Mean probing pocket depth, clinical attachment loss, plaque index and bleeding on probing scores were significantly higher in the chronic and generalized aggressive periodontitis groups, than in the control group ($p < 0.0001$). No significant differences were detected in these clinical measurements between the two periodontitis groups.

Analysis of sTREM-1 levels in saliva

Saliva samples were collected from 59 subjects (control: n = 18, chronic periodontitis: n = 20, generalized aggressive periodontitis: n = 21). The concentrations of sTREM-1 in these samples were further analyzed by ELISA. sTREM-1 was detected in all of the samples. The mean concentrations were 384.60 ± 115.81 pg/ml in the control group, 1272.38 ± 138.91 pg/ml in chronic periodontitis (3.3-fold higher than the control) and 2179.95 ± 306.14 pg/ml in generalized aggressive periodontitis (5.6-fold higher than the control) (Fig. 1A). The differences between the control group and either form of periodontitis were statistically significant, whereas the difference between the two forms of periodontitis was not. Since salivary flow rate may reflect differences in composition, the salivary sTREM-1 concentrations were also corrected for total protein content. It was found that total protein concentrations were 1.73 ± 0.28 mg/ml, 2.03 ± 0.31 mg/ml and 2.49 ± 0.43 mg/ml, in the control group, chronic periodontitis and generalized aggressive periodontitis, respectively. Accordingly, the calibrated-to-protein salivary levels of sTREM-1 were 212.18 ± 32.23 pg/mg, 914.45 ± 170.99 pg/mg and 1127.16 ± 133.63 pg/mg, in the control group, chronic periodontitis (4.3-fold higher than control) and generalized aggressive periodontitis (5.3-fold higher than control), respectively (Fig. 1B). The differences between the control group and chronic or generalized aggressive periodontitis were statistically significant ($p < 0.0001$), whereas the difference between these two forms of periodontitis was not. Salivary sTREM-1 levels (both pg/ml and pg/mg protein) correlated positively ($p < 0.01$) with all full mouth clinical parameters measured (Table 2). There were no significant differences in salivary sTREM-1 concentrations or calibrated-to-protein amounts between smokers and non-smokers. There was also a positive correlation between IL-1β and sTREM-1 concentrations and calibrated-to-

protein amounts in saliva (r = 0.587 and r = 0.565, respectively, $p < 0.05$). The analysis of IL-1β levels in saliva is provided in the Appendix.

Analysis of sTREM-1 levels in serum

Serum samples were available from 62 subjects (control: n = 20, chronic periodontitis: n = 22, generalized aggressive periodontitis: n = 20) and analyzed by ELISA for the concentrations of sTREM-1, which was detected in all samples. These concentrations were 306.49 ± 18.65 pg/ml in the control group, 536.89 ± 43.98 pg/ml in chronic periodontitis and 621.7 ± 54.35 pg/ml in generalized aggressive periodontitis (Fig. 2). These differences represent a 1.75-fold increase in chronic periodontitis and a 2-fold increase in generalized aggressive periodontitis, compared to the control group. The differences between the control group and either form of periodontitis were statistically significant ($p < 0.0001$), whereas there were no differences between the two forms of periodontitis. Serum TREM-1 levels also positively correlated ($p < 0.01$) with all full mouth clinical parameters (Table 2). There was no significant difference ($p > 0.05$) in sTREM-1 serum concentrations between smokers and non-smokers.

Correlation analysis between saliva and serum sTREM-1 levels

The potential correlation between sTREM-1 levels in saliva and serum was also investigated, in the 58 subjects with the available matched samples, a positive correlation between sTREM-1 concentrations in serum and saliva was revealed (r = 0.48, $p < 0.0001$).

DISCUSSION

The present study is the first to investigate the oral and systemic levels of sTREM-1 in periodontal disease, by analysing its concentrations in serum and saliva, respectively. The results demonstrate that the levels of sTREM-1 in saliva and serum are significantly higher in patients with chronic and generalized aggressive periodontitis, compared to subjects without periodontitis. There are no differences between these two forms of periodontitis. Moreover, a positive correlation is revealed between clinical periodontal measurements and levels of sTREM-1. This indicates that the occurrence and severity of periodontitis matches with elevated oral and systemic sTREM-1 levels. Taken the involvement of this molecule in the propagation of the local and systemic inflammatory response, these findings could provide a further link between periodontitis and systemic inflammation (Hasturk et al., 2012).

A number of systemic biomarkers have been studied to assess their relevance to periodontal health and disease. For instance, C-reactive protein (CRP), a systemic marker of the acute phase inflammatory response, has been measured in saliva and serum, and can differentiate periodontal health from periodontal disease, as its levels are elevated in disease (Christodoulides et al., 2005; D'Aiuto et al., 2004). When compared to CRP or procalcitonin, a marker that can differentiate between infectious and non-infectious inflammation, serum and urine sTREM-1 shows higher sensitivity and specificity for detecting systemic inflammation in early bacterial sepsis (Su et al., 2012). In this respect, sTREM-1 could well serve as a biomarker representative of the inflammatory state of the infected periodontal tissues. The nature of periodontitis as a focal infection and its well documented association with systemic inflammation, provide a good rationale for studying sTREM-1 systemic responses in this disease. Indeed, the present data demonstrates a significant increase of salivary and serum

sTREM-1 in periodontitis compared to non-periodontitis, as well as a positive correlation with disease severity. Moreover, the data provides evidence of a positive correlation between sTREM-1 and IL-1β levels in saliva. This association is in line with *in vitro* findings that TREM-1 activation enhances IL-1β production (Bostanci et al., 2011), although the present approach can not directly reveal a cause-effect relationship between the two molecules in saliva. Saliva in particular is a favourable biological analyte for monitoring oral and systemic disease biomarkers, due to its non-invasive collection and natural presence in the totality of the oral cavity (Giannobile et al., 2009; Ramseier et al., 2009; Zhang et al., 2009). Hence, the 3-fold to 6-fold increase of sTREM-1 salivary levels in periodontitis can denote the magnitude of pathological regulation, opening the possibility for the use of sTREM-1 as a quantitative biomarker for undiagnosed periodontal disease. At this point, one should take also under consideration that proteinases produced by oral bacteria, particularly the ones associated with periodontitis, could well degrade sTREM-1 released in saliva. This would mean that the original production of sTREM-1 may be greater than what is eventually is detected in the salivary environment.

It is likely that inflammatory biomarkers of oral disease would be considerably diluted in serum, compared to saliva. This is also reflected in the present study, whereby sTREM-1 concentrations in periodontitis were by 2-fold higher in saliva than in serum. As periodontal disease can be a mediator of systemic inflammation and a modifier of the effect of the association with systemic disease, such as diabetes mellitus (Lalla and Papapanou, 2011), sTREM-1 could provide a risk indicator for systemic inflammation. In the present study, the periodontitis patients were otherwise systemically healthy, with no other indication of an inflammatory or infectious process, and demonstrated almost 2-fold higher sTREM-1 serum levels, compared to

subjects without periodontitis. Hence, once could postulate that the occurrence of periodontitis alone increases by 2-fold the circulating levels of sTREM-1, and consequently any biological effect that this factor may have on the systemic level. Whether the increased presence of sTREM-1 in the serum of periodontitis patients results from bacteraemic challenge of circulating leukocytes by periodontal pathogens, or represents a "leakage" of locally produced sTREM-1 into the circulation remains unclear. Finally, it is worth noting that as salivary and serum sTREM-1 does not appear to be affected by smoking, unlike other inflammatory markers (i.e. CRP and several cytokines), it may prove to be a useful biomarker.

Collectively, the present study shows an increase of oral and systemic levels of sTREM-1 in periodontitis. The two main limitations of the study have been its cross-sectional nature, which can only confer an association between sTREM-1 and periodontitis, but can not reveal a mechanistic insight or a predictive value for the disease, and the relatively small sample size. Whether sTREM-1 release constitutes a mechanism that contributes to the progression of the disease, or a protective inflammatory reaction to emerging bacterial infection, remains to be elucidated. Whichever the case, the increased presence of this molecule may stand as a biomarker of underlying periodontal disease. This can have diagnostic implications in the detection of untreated periodontitis, but could also denote an increase in the systemic inflammatory burden, in the presence of the disease. In either case, the physiological and/or pathological roles of sTREM-1 in periodontal inflammation need to be further enlightened in order to justify its potential value as an indicative or predictive oral biomarker of the disease, or as an indicator of the association between periodontal disease and systemic inflammation.

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TABLES

Table 1. Full mouth clinical periodontal measurements (mean ± standard deviations) of the three study groups.

Clinical parameters	Control (n = 20)	Chronic Periodontitis (n = 22)	Generalized Aggressive Periodontitis (n = 21)
Probing pocket depth (mm)	1.80 ± 0.26	4.95 ± 0.37 *	5.32 ± 0.77 *
Clinical attachment loss (mm)	1.83 ± 0.28	5.56 ± 0.67 *	6.02 ± 1.07 *
Plaque index	0.85 ± 0.39	2.35 ± 0.48 *	2.09 ± 0.44 *
Bleeding on probing (%)	25 ± 15	74 ± 41 *	66 ± 21 *

*$p < 0.0001$

Table 2. Spearman's rank correlation analysis between full mouth clinical periodontal parameters and TREM-1 levels saliva and serum.

Clinical parameters	Serum (pg/ml)	Saliva (pg/ml)	Saliva (pg/mg)
Probing pocket depth	r = 0.674 *	r = 0.672 *	r = 0.694 *
Clinical attachment loss	r = 0.725 *	r = 0.640 *	r = 0.725 *
Plaque index	r = 0.525 *	r = 0.544 *	r = 0.589 *
Bleeding on probing	r = 0.427 *	r = 0.674 *	r = 0.583 *

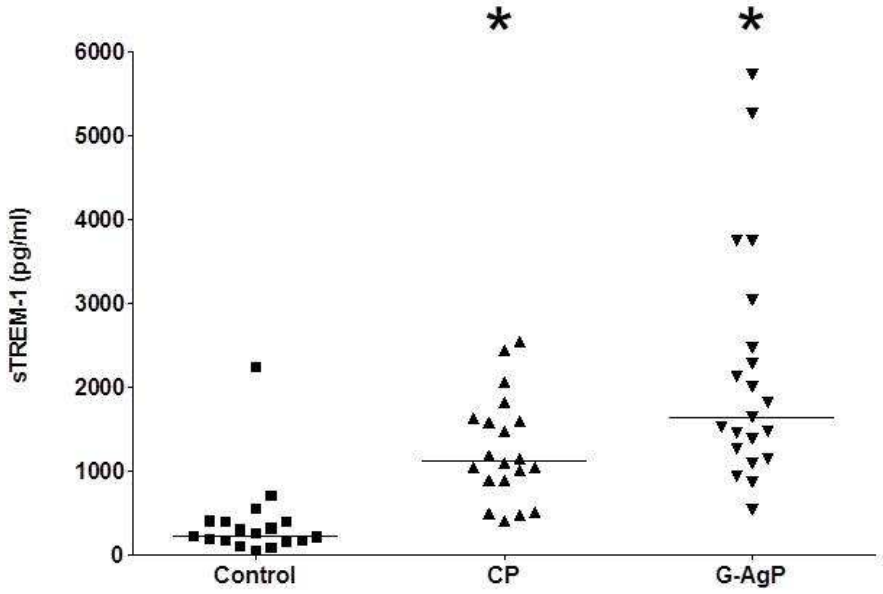
* $p < 0.01$

FIGURE LEGENDS

Figure 1. Distribution of TREM-1 levels in saliva from subjects without periodontitis (control) (n = 18), with chronic periodontitis (n = 20) and with generalized aggressive periodontitis (n = 21) subjects. The individual values represent salivary (A) concentrations (in pg/ml) or (B) amounts (in pg/mg of protein) of TREM-1 in each subject. The horizontal lines indicate median values. The asterisk represents significant difference compared to the control group (* $p < 0.0001$). The difference between the chronic periodontitis and generalized aggressive periodontitis groups was not significant.

Figure 2. Distribution of TREM-1 levels in serum from subjects without periodontitis (the control group) (n = 20), chronic periodontitis (n = 22) and generalized aggressive periodontitis (n = 20) subjects. The individual values represent serum concentrations (in pg/ml) of TREM-1 in each subject. The horizontal lines indicate median values. The asterisk represents significant difference compared to the control group (* $p < 0.0001$). The difference between the chronic and generalized aggressive periodontitis groups was not significant.

Fig. 1A


*
*
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5000
4000
3000
2000
1000
0
sTREM-1 (pg/ml)
Control
CP
G-AgP

Fig. 1B

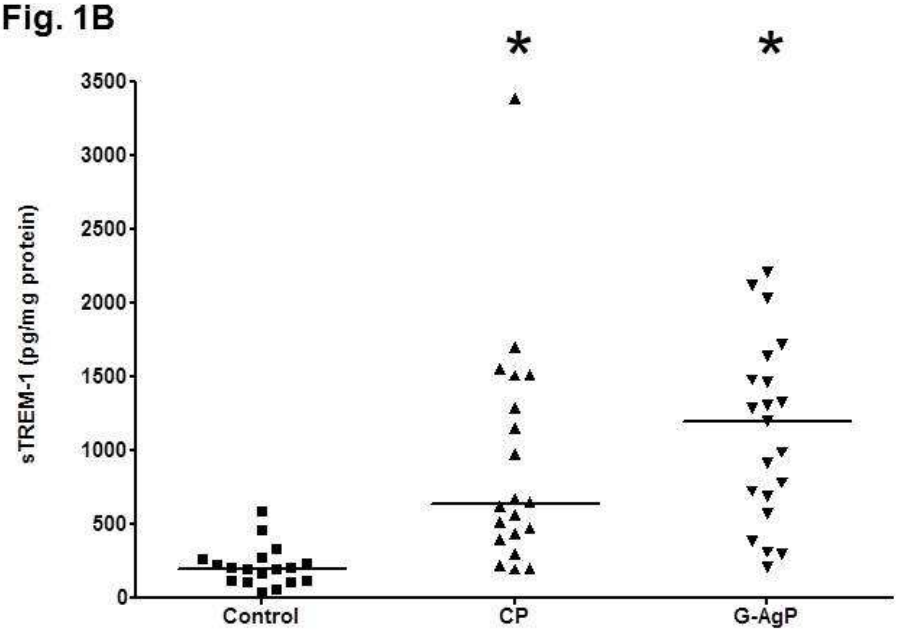
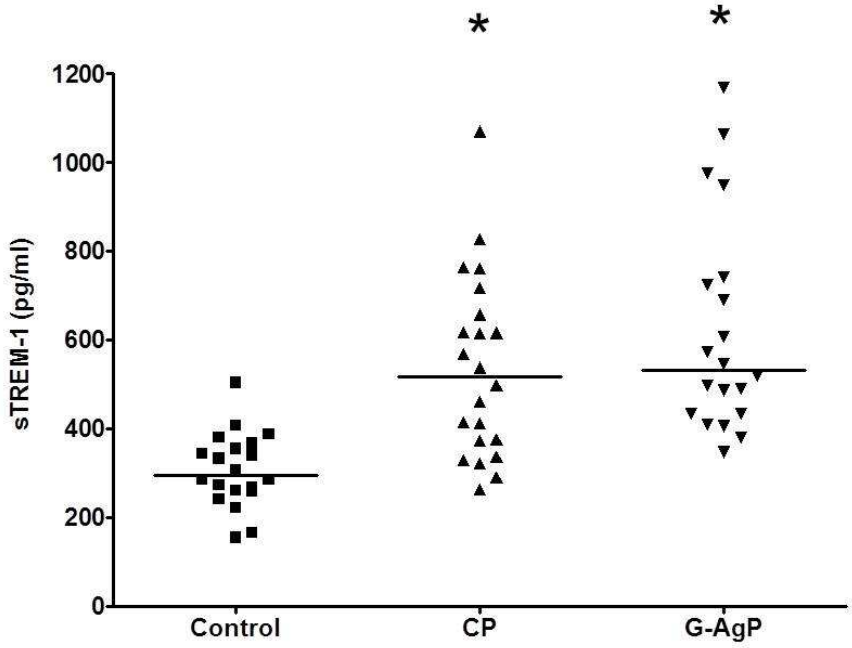

*
*
3500
3000
2500
2000
1500
1000
500
0
sTREM-1 (pg/mg protein)
Control
CP
G-AgP

Fig. 2



APPENDICES

Materials and Methods

Patient demographics and clinical measures

The periodontal status of each patient was assessed by a single calibrated examiner having experience in clinical trials (O.V.O). Full mouth clinical periodontal examination included measurement of probing pocket depth, clinical attachment level at six sites around each tooth with a manual probe, full-mouth dichotomous presence of bleeding on probing and plaque index (Loe, 1967). The following values represent means ± standard deviation. The control group consisted of 6 females and 14 males

(from 21 to 64 years, mean age 36.0 ± 2.6 years) with varying levels of gingival inflammation as indicated by bleeding on probing scores, but no radiographic evidence of alveolar bone loss (i.e., distance between the cemento-enamel junction and bone crest ≤3 mm at >95% of the proximal tooth sites). This control group is collectively considered as the "non-periodontitis" group in the context of this study. The chronic periodontitis group included 13 females and 9 males (from 33 to 62 years, mean age of 44.1 ± 7.7 years). They had at least 4 non-adjacent teeth with sites of clinical attachment loss ≥5 mm, which was commensurate with the amount of plaque accumulation, and probing pocket depth ≥6 mm. Full mouth bleeding on probing was more than 50%. The generalized aggressive periodontitis group consisted of 12 females and 9 males (from 23 to 42 years, mean age 34.2 ± 5.3 years). These patients demonstrated a generalized pattern of severe destruction and clinical attachment loss ≥5 mm and pocket depth ≥6 mm on eight or more teeth, at least three of these were other than central incisors or first molars. All subjects had at least 20 teeth in their mouth. The extent and severity of bone support and/or osseous lesions were evaluated radiographically in each patient. The distribution of smokers was 4/20 in the non-periodontitis group, 7/22 in the chronic periodontitis group, and 7/21 in the generalized aggressive periodontitis group.

Analysis of TREM-1 and IL-1β levels by ELISA

The levels of sTREM-1 in saliva or serum, and the levels of IL-1β in saliva were measured by a commercially available specific enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The absorbance at 450 nm was measured using a microplate reader (Epoch, BioTek, Luzern, Switzerland), with a wavelength correction set at 570 nm to subtract

background. A standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed. All samples were run in duplicate wells. The sensitivity of the sTREM-1 ELISA assay was 46 pg/ml, whereas that of the IL-1β ELISA assay was 1.7 pg/ml. The concentrations of sTREM-1 in saliva were also expressed as pg/mg of protein, after normalization against total protein levels. The total protein content of saliva was quantified with the BCA Protein Assay Kit (Thermo Scientific Pierce, Switzerland) as mg/ml.

Results

Analysis of IL-1β levels in saliva

IL-1β concentrations were significantly higher in chronic periodontitis (163.62 ± 28.59 pg/ml, $p < 0.05$) and generalized aggressive periodontitis (334.34 ± 91.73 pg/ml, $p < 0.0001$), compared to the control group (97.77 ± 14.28 pg/ml). The difference between the two forms of periodontitis was also significant ($p = 0.03$). When the calibrated-to-protein IL-1β levels were considered, these were significantly higher in chronic periodontitis (107.28 ± 17.63 pg/mg, $p = 0.05$) and generalized aggressive periodontitis (151.61 ± 31.24 pg/mg, $p = 0.02$), compared to the control group (64.39 ± 10.18 pg/mg).

References

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